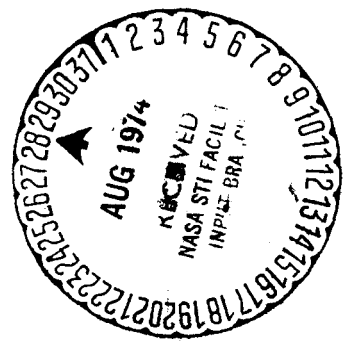

PROGRESS REPORT

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A Rapidly Recording Microspectrophotometer

by

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ABSTRACT

A Microspectrophotometer M-5 of increased sensitivity has been constructed to record absorption spectra rapidly in a single sweep from the ultraviolet through the visible, from 2500 to 8000 Å. The M-5 records from single cells and from cell organelles of the order of 0.5 μ^2, using minimum light. This report reviews the general design, the nature of the component parts, and the performance of the instrument.

INTRODUCTION

The precise composition of living cells is difficult to determine and the functional chemistry is even more difficult to unravel. To study the chemistry of living cells and their organelles, e.g., nucleus, mitochondria, chromatophores, and photoreceptors, microanalytical instrumentation is necessary. The need for spectroscopic instrumentation was recognized in the 1930's by Casperson, who initiated the development of microspectrophotometry for ultraviolet absorption studies of the cell nucleus (1,2). Recording microspectrophotometers have since been developed and applied to biological studies (3-6). These microspectrophotometers, with the exception of two commercial instruments (7), are laboratory built. Low-light-level recording microspectrophotometers have been designed by Liebman and Entine (5) and a microfluorospectrophotometer by Runge (8); these were applied to the identification of visual pigments of the eye and of porphyrins. The microspectrophotometers employ either split- or dual-beam optical systems with mechanical beam chopping devices, voltage regulated photomultiplier tubes with feedback mechanisms, and automatic recording outputs.

The recording microspectrophotometer M-5 described here is a new instrument designed to extend the usefulness of our earlier models (6) by: increasing the optical resolution; increasing the sensitivity in the ultraviolet, visible, and the near infrared; operating at low light levels (10^4 photons per second or a total of 2×10^5 photons for a measurement); and reducing the noise in the system.

GENERAL DESCRIPTION

The Microspectrophotometer M-5 is shown in the photograph, Fig. 1. The principal components are illustrated in Figs. 2-5. Fig. 2 shows the chopper, Fig. 3 the optical system, Fig. 4 the electronic diagram, and Fig. 5 the electronic circuitry. The characteristics of the components are summarized in Table I.

In operation, light from a tungsten or a xenon lamp passes through a Canalco grating monochromator with variable slits to a specially designed chopper (Fig. 2). The chopper divides the beam of light into two beams which pulse alternately in time. Each beam is on 40% of the time and is pulsed 480 times per second. The two beams of light are directed into a condenser of a microscope, which is an objective lens of 100 X or less. Its purpose is to focus the two light beams as two spots of light on the specimen slide. The slide is moved to locate the specimen area to be investigated over one of the spots while the other spot is used as a reference. The light is then collected and focused by the objective, and a simple quartz lens (L_3) focused on the back diaphragm of the objective directs all the light onto the cathode of the EMI 9558QA photomultiplier tube.

For viewing the specimen and locating it with respect to the spots of light, a swingout prism directs the light to the binocular eyepieces. A rotating reflecting blade serves to direct light from the microscope illuminator into the light path to illuminate the background which can then be viewed simultaneously with the monochromatic spots of light. When the swingout prism is in position for a measurement the rotating reflecting blade is automatically stopped

so as not to interrupt the monochromatic light path and only the monochromatic light goes to the photomultiplier.

The photomultiplier output current (Fig. 4) travels through a field effect transistor (load F.E.T.) which is the element used in conjunction with a DC feedback amplifier (AGC) to automatically control the photomultiplier output voltage. The signal is amplified by the AC amplifiers after which the DC level is restored and the signal is synchronously switched into separate RC smoothing circuits. Synchronization signals are supplied by a light source and photo-transistor arrangement at the chopper. The smoothed reference signal is then used in the feedback circuit to keep the average level of the reference signal at the amplifier output a constant.

The average level of the sample signal is then directly proportional to the absorption of the specimen; this signal is recorded on the chart recorder which plots the percent absorption versus wavelength.

DESIGN AND COMPONENTS

Light source. Two light sources can be used, either the xenon lamp, which has a useful spectrum of light from 2250 to 14000 Å, or the tungsten quartz iodine lamp, which has a more stable output and a useful spectrum of light from 2000 Å to beyond 10000 Å. The quartz iodine lamp output is much lower than that of the xenon lamp in the ultraviolet.

Monochromator. The monochromator used is a Canalco rapid-scan which uses a diffraction grating as the dispersing element. It is calibrated from 2000 to 8000 Å which limits the spectral range of the microspectrophotometer. For normal operation a slit width of 0.5 millimeter is used giving a half bandwidth of 20 Å. The ten-speed mechanical drive mechanism has been modified to scan the spectrum at rates from 2 to 2000 Å per second. A wavelength-indicating disc has been fitted to the main shaft with a miniature light source and a photocell plus a transistor amplifier. This arrangement produces a signal which is "on" for 500 Å and "off" for 500 Å with pulses for each specific 1000 Å interval; this signal triggers the event marker on the chart recorder.

Chopper. The chopper (Fig. 2) was designed and built in the laboratory to take a single monochromatic beam of light and to produce two identical beams of light which are spaced about 0.5 inch apart. The beams are pulsing alternately in time, each "on" about 40% of the total time for each cycle. Mounted on the housing of the chopper is a light source and phototransistor arrangement which produces signals synchronized with the light pulses for switching the amplifier output. The chopper is, then, also a beam-splitter and a synchronous signal generator.

The accuracy in measuring the relative percentage of absorption depends on the relative heights of the two pulses; the sample and reference areas are not measured simultaneously but alternately in time. Therefore, if the overall accuracy is to stay within one percent, any change in the relative height of the pulses due to spectral quantum efficiency of the

photocathode, the spectral distribution of the light source, or the gain of the amplifiers should be within one percent during the time for one pair of pulses. Consequently, a short scanning time requires a correspondingly short pulse pair-time, which demands the development of a precision, high-speed chopper and special electronic circuits in the amplifier.

The chopper consists of a set of four rotating mirrors and one stationary mirror. There are eight posts situated on the chopper so that they interrupt the light after each pulse for 10% of the cycle time. The mirrors are front surface mirrors and flat to a quarter wavelength. They vary somewhat in mass, so provision was made for balancing the rotor after installation of the mirrors.

The two beams of light, sample and reference, leave the chopper unit at about 90° with respect to the single entrance beam. Both are directed into the microscope by aligning the chopper housing and the stationary mirror with the optic axis of the microscope and the monochromator.

There are two apertures in the system through which the light passes to form the beams of light. One is a variable slit arrangement which adjusts the height and width of a rectangular spot of light. The other is a fixed aperture located at the entrance to the chopper unit; this serves to aid in the alignment of the system as well as to limit the maximum diameter of the beam. The microscope condenser, which is used to bring the beams into focus as spots of light on the

slide, is focused on the variable slits. With the 100 X condenser, the maximum dimensions of the spots are 8 μ at the chopper. The minimum size of the spots for practical purposes is on the order of 0.5 μ.

Optics. Two types of optics can be used, either reflecting or refracting. The focal length of the reflecting optics has no dependency on wavelength; consequently, the focal point does not change with wavelength. The reflecting optics lack resolving power in the visible spectrum as compared to the refracting optics. When high resolution is required, the Zeiss ultrafluar optics are used, even though they are limited in their spectral range. Although the manufacturer specifies them to be correct between 2400 and 7000 Å, a side shift of about three microns was found for a spot near the edge of the field, using a 32 X ultrafluar as the objective and a 100 X as the condenser. A spot of light positioned in the center of the field does not shift; therefore the sample spot of light is positioned in the center of the field and the reference spot on the edge. For locating and positioning the specimen the contrast can be improved either by lowering or by diaphragming the condenser.

Microscope. An American Optical Co. microscope was modified to facilitate alignment, to permit accurate adjustments, and to make it usable in the ultraviolet. To shield against ambient light, a microswitch was installed on the swingout prism slide bar which operates a relay that cuts the power to the background illuminator, removes AC from the winding of the hysteresis motor and applies DC.

The reflecting blade is stopped out of the monochromatic light path and therefore prevents the background illumination from entering the photomultiplier.

To facilitate location of the specimen with respect to the spots of light the stage can be rotated as well as moved with the ordinary degree of freedom of the mechanical stage.

A thermoelectric temperature control device is fitted to the stage and the temperature of the specimen can be controlled between -20°C and 100°C.

To provide a good foundation for the optical components, a honeycomb sandwich platform (48 x 24 x 1.625 inches) was supplied by Hexcel Corporation, Havre de Grace, Md. Aluminum plates 0.125 inches thick are glued to each side of the honeycomb. This is necessary for threading holes to secure the components to the platform, which serves as a light-weight, rigid surface to secure and align the whole optical system.

Photomultiplier. An EMI 9558QA photomultiplier tube with a diameter of 4.5 cm is used as the sensor. The cathode material is SbNaKCS (S-20) on fused silica. The photocathode sensitivity is about 140 amps/lumen; the overall sensitivity of the photomultiplier tube is 200 amps/lumen. The photomultiplier tube with a quartz window has a spectral response between 1650 and 8400 Å. The photomultiplier housing is an EAO cooling housing fitted with an EOVac (evacuated) quartz window. The cooling housing has a potted voltage divider and a selector switch for four different load resistances and an infinite resistance position. The

dark current is 6×10^{-9} amps at 20°C. The ratio of the high sensitivity to low dark current means an extremely high ratio of signal to noise. Cooling the tube with liquid nitrogen improves the signal to noise ratio about 100 times.

Circuitry. All the circuitry following the photomultiplier except for the high voltage supply is transistorized (Fig. 5), including the Sanborn oscillographic chart recorder. The automatic gain control is accomplished by controlling the photomultiplier load resistance, which is linear for drain to source voltages with magnitudes less than 50 millivolts. The magnitude of the resistance can be varied over a range of about 5000 to 1 simply by varying the gate to source voltage on the transistor; therefore the multiplier voltage output can be held constant over this range of output current. After the signal is sufficiently amplified the DC level of the signal must be restored, since the amplifiers are AC coupled. The DC level is restored by a clamping circuit which shorts the signal during the dark time between pulses. This is accomplished by connecting the signal at the amplifier output to the drain of a field effect transistor; the source is grounded. The gate, then, receives a pulse of about 70 microseconds duration during the 100 microsecond dark time of the signal. The pulse at the gate is shaped by a flip flop circuit which is in turn triggered by the synchronized signal from the chopper.

The clamped signal is then sent to the electronic switch which consists of two field effect transistors also triggered by the chopper signal. The function of this electronic switch is to separate the sample and reference signals into two separate channels. The sample

channel consists of an RC smoothing circuit and the chart recorder; the reference channel consists of the DC feedback system which holds the reference constant.

The sample and reference channels can be electronically switched on the control panel so that either monochromatic light spot can be used as the sample beam. Also on the control panel are selectors for the response time of each channel and a DC volt meter which monitors the reference feedback voltage.

DESIGN THEORY

The advantage of keeping the output level of the reference channel constant is demonstrated in Fig. 6. R_i is the average level of the reference signal current from the photomultiplier. S_i is the average level of the sample signal. These signals may be considered separately even though there is a single photomultiplier because they are trains of pulses interlaced in time and the amplifier output is switched synchronously to separate these two trains (9).

$R_i \propto I(\lambda)A(\lambda)\varepsilon(\lambda)$ where $I(\lambda)$ is the light flux, as a function of wavelength and $A(\lambda)$ is the transmittance of the optics for the reference beam. $\varepsilon(\lambda)$ is the photocathode sensitivity.

$S_i \propto I(\lambda)\ A(\lambda)\ \varepsilon(\lambda)S(\lambda)$ where $S(\lambda)$ is the transmittance of the sample area as a function of wavelength. S_i differs from R_i only by the additional factor $S(\lambda)$ because the sample beam is identical to the reference beam except for the presence of the sample.

Therefore $R_o \propto I(\lambda)A(\lambda)\varepsilon(\lambda)G(\lambda)$ where R_o is the output voltage of the amplifier reference channel and $G(\lambda)$ is the amplifier gain.

The feedback is used to keep R_o constant; therefore the gain changes so that

$$G(\lambda) \propto \frac{1}{I(\lambda)\, A(\lambda)\, \varepsilon(\lambda)} .$$

But since the amplifier gain is the same for both sample and reference signals we have

$$S_o \propto I(\lambda)\, A(\lambda)\, \varepsilon(\lambda)\, G(\lambda)\, S(\lambda) = S(\lambda)$$

Therefore, the average level of S_o at any given wavelength is directly proportional to the transmittance of the sample area at that wavelength.

Photomultiplier S/N. The overall speed of response of the system for a given degree of accuracy is limited principally by three random processes. These are: thermionic emission by the photocathode, the photon arrival at the photocathode, and the electron multiplication at the dynodes. The noise current due to thermionic emission may be reduced to a negligible amount by cooling the photomultiplier to dry ice or liquid nitrogen temperatures. The noise, then, is due almost entirely to the randomness in the light flux and in the dynode electron multiplication. These two noise sources determine the light levels required for any desired system output signal to noise ratio and speed of response.

For all light levels and photomultiplier currents normally encountered Maxwell Boltzmann statistics apply and therefore the well-known Johnson noise expression may be used for calculating the noise currents associated with a given DC current (10).

The following will be a derivation of the signal to noise ratio to be expected from a cooled photomultiplier in terms of the input light flux. The analysis is done for a DC light input but is easily extended to a chopped light input.

For analysis purposes, the light flux given by L photons/sec. may be represented by an equivalent current I_L where $I_L = qL$ and q is the electronic charge. The measured RMS noise current squared, i_L^2, is then given by the expression for Johnson noise and equals $2q\, I_L\, \Delta f$ where Δf is the overall bandwidth of the measuring system. The signal to noise power ratio in the equivalent input current is $(S/N)_L$ which may then be represented by

$$\frac{I_L^2}{i_L^2} = \frac{I_L}{2q\Delta f} .$$

If ε is the photocathode quantum efficiency then $I_1 = \varepsilon I_L$. However, i_1^2 now is the sum of two terms. The first is the Johnson noise term due to I_1 which equals $2qI_1\Delta f$ which occurs because the electron emission from the photocathode due to the DC current I_L is random. The second term equals $\varepsilon^2 i_L^2$ which equals $\varepsilon^2 2qI_L\Delta f$ and is due to the multiplication of the noise current already present in the input current. Therefore

$$\begin{aligned} i_1^2 &= 2qI_1\Delta f + \varepsilon^2 2qI_L\Delta f \\ &= 2q\varepsilon I_L\Delta f(1+\varepsilon) \end{aligned}$$

Therefore $(S/N)_1$, the signal to noise ratio at the input to the first dynode, equals

$$\frac{\varepsilon^2 I_L^2}{2q\varepsilon I_L\Delta f(1+\varepsilon)} = \frac{\varepsilon}{1+\varepsilon}\,(S/N)_L .$$

This expression shows that the higher the quantum efficiency, the smaller the reduction in the signal to noise ratio. For work in the range from 2000 to 8000 Å the S-20 photocathode has a higher quantum efficiency than any other photocathode available.

The derivation for the signal to noise ratio at the input to the second dynode is as follows. The DC current input $I_2 = \varepsilon\sigma I_L$ where σ is the dynode electron multiplication factor. Then

$$i_2^2 = 2q\varepsilon\sigma I_L \Delta f + \sigma^2 i_1^2.$$

After taking the ratio $\frac{I_2^2}{i_2^2}$ we arrive at $(S/N)_2 = \frac{\sigma\varepsilon}{[1 + \sigma(1+\varepsilon)]} (S/N)_L$

The signal to noise ratios at the rest of the dynodes may be derived in a similar manner. A typical value for ε is 0.1 and for σ is 3.5. Therefore

$$(S/N)_1 = \frac{\varepsilon}{1+\varepsilon} (S/N)_L = 0.09 \ (S/N)_L$$

$$(S/N)_2 = \frac{\sigma\varepsilon}{[1+ \sigma(1+\varepsilon)]} (S/N)_L = 0.072 \ (S/N)_L$$

$$(S/N)_3 = \frac{\sigma^2\varepsilon}{1+\sigma[1+\sigma(1+\varepsilon)]} (S/N)_L = 0.068 \ (S/N)_L$$

$$(S/N)_4 = 0.067 \ (S/N)_L$$

This approaches a limiting value. $(S/N)_o$, the signal to noise ratio of the output, is very nearly equal to the signal to noise ratio available after the first dynode. Therefore the gain of the photo-multiplier may be as high as is needed to overcome the noise in the amplifier so that the signal to noise ratio of the output of the photomultiplier, amplifier, smoothing circuit system can be nearly equal to $(S/N)_o$.

The overall signal to noise ratio for the system for $\epsilon = 0.1$ and $\sigma = 3.5$ is given by:

$$(S/N)_o \simeq 0.07\ (S/N)_L = 0.07\ \frac{I_L}{2q\Delta f}$$

since $I_L = qL$

$$(S/N)_o = 0.07\ \frac{L}{2\Delta f} \quad .$$

This equation also applies in the case of a system using chopped light, by the fact that L is the average light flux in photons/sec. It relates the photomultiplier parameters to: the system response speed (which is approximately equal to $\frac{1}{\Delta f}$), the average light flux L, and the output signal to noise ratio.

A complete report of the design of the M-5 was submitted to Review of Scientific Instrumentation for consideration for publication in August, 1967.

FUTURE CONSIDERATIONS

The Microspectrophotometer M-5 is a working instrument. It can serve as a prototype for development of a miniaturized instrument which hopefully will be used for extraterrestial exploration and for oceanography. Efforts in this direction are now being made.

TABLE I

Microspectrophotometer Component Characteristics

Component	Characteristics
Light sources	
Xenon lamp	Osram 150W; Sola power supply
Tungsten lamp	Quartz iodine; 100W, DC
Monochromator	
Wavelength span	2000 to 8000 Å
Diffraction grating	blaze at 5000 A; 600 groves/mm
Dispersion	40 Å half-bandwidth per mm slit width
Scanning mechanism	2 to 2000 Å per second; 10 steps
Background illuminator	
Tungsten ribbon filament	6V, 18A, AC
Reflecting blade	3600 rpm; 75 V AC or 50 V DC
Microscope	American Optics Co.
Zeiss ultrafluar lens	condenser 100 X; objectives 100 X and 32 X; 2400 to 7000 Å
AO reflecting lens	condenser 50 X; objective 50 X
Photomultiplier	EMI 9558QA
Spectral response	S-20; 1650 to 8400 Å
Quantum efficiency	>20%, 1800 to 4200 Å; 10% at 5600 Å
Supply voltage	Hammer N401 500-1800 volt
DC dark current	6×10^{-9} amps at 20°C
Anode load	2N2386 transistor (F.E.T.)
Load resistance range	5000 to 1 linear for voltages <50MV
Cooler	EOA PM 102; -80°C
Preamp	Mounted on EOA PM 102
Amplifier chain	gain 3×10^4
Response time	0.01 to 1.0 seconds
Reference feedback	DC; gain 1000
Recorder	Sanborn 7701A
Preamp	8803A
Sensitivity setting	1 volt full span
Speeds	0.5 to 50 mm/sec or 0.5 to 50 mm/min

LEGENDS FOR FIGURES

Fig. 1 Microspectrophotometer M-5

a. Lamp house; b. grating monochromator; c. chopper; d. photomultiplier tube housing; e. charter recorder.

Fig. 2 a. Component parts of the chopper.

b. Schematic showing the chopper's function as a beam splitter.

Fig. 3 Microspectrophotometer optical system.

Fig. 4 Microspectrophotometer electronic block diagram.

Fig. 5 Microspectrophotometer electronic schematic.

Fig. 6 Automatic ratio operation.

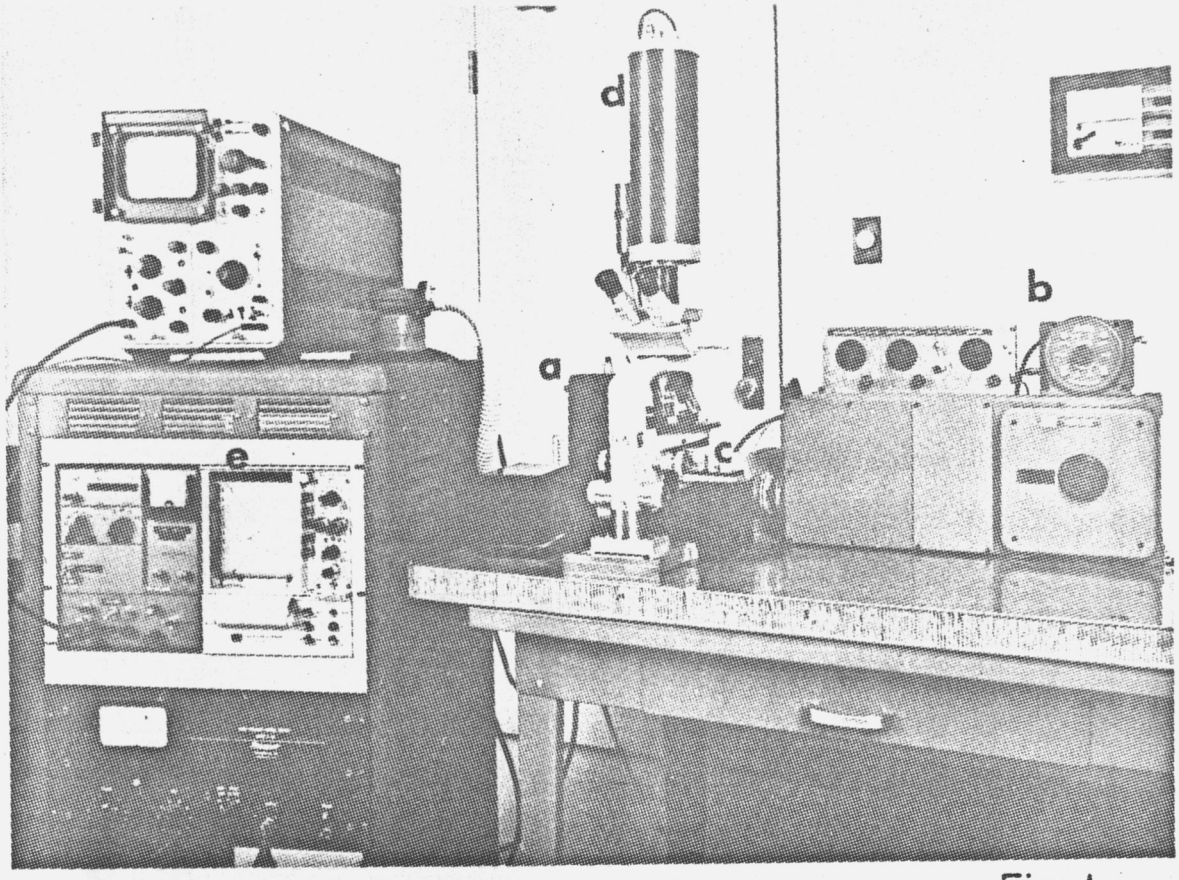
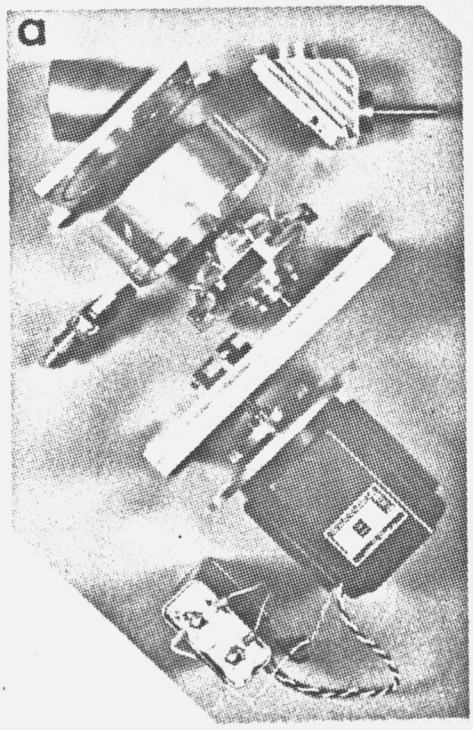

d
a
b
c
e

Fig. 1


a

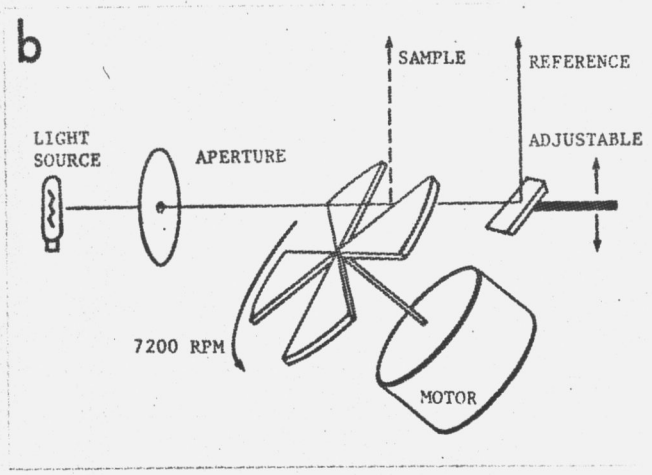

b
SAMPLE
REFERENCE
LIGHT SOURCE
APERTURE
ADJUSTABLE
7200 RPM
MOTOR

Fig. 2

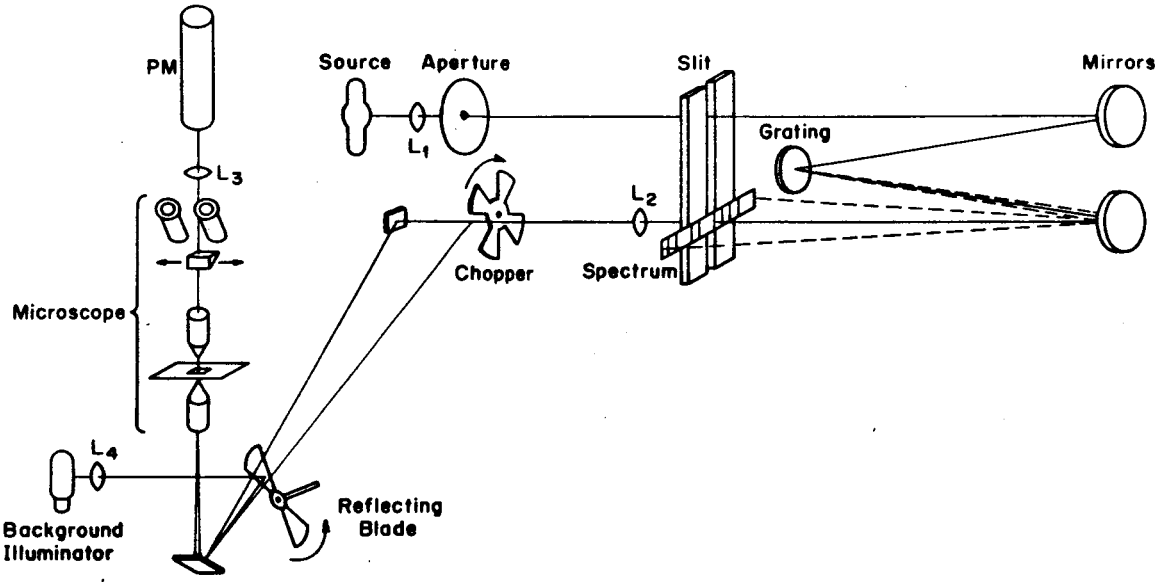
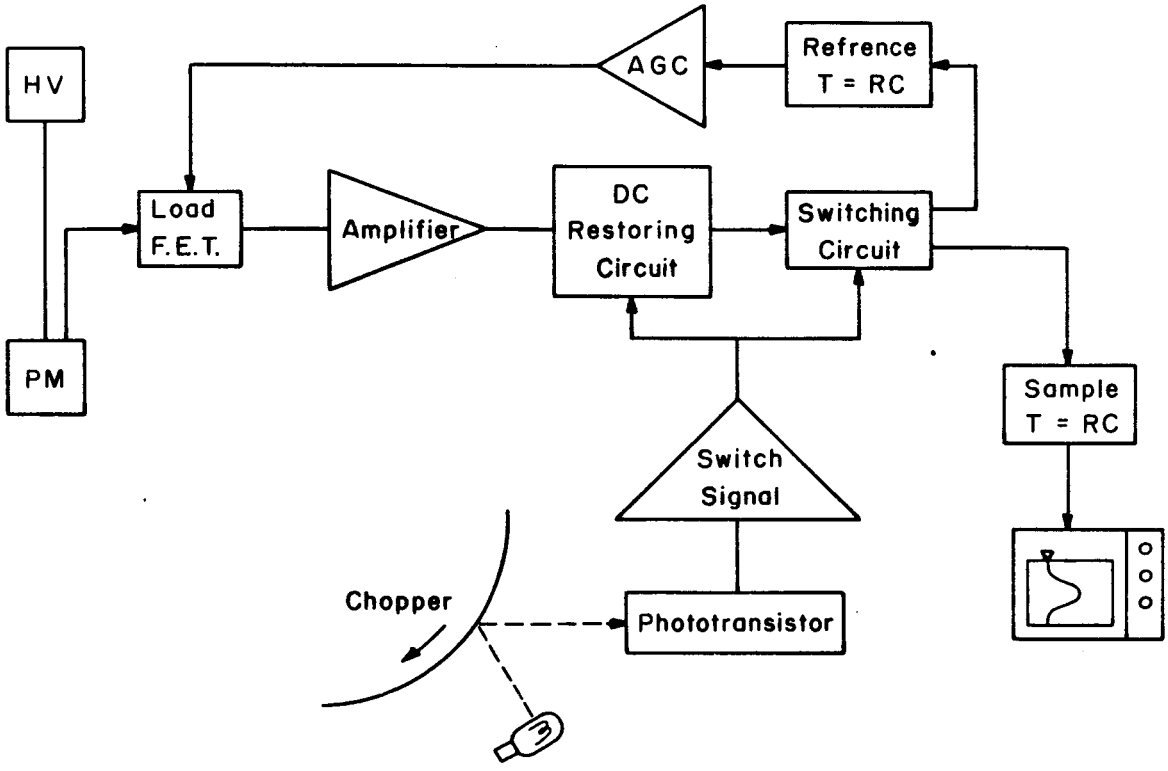


FIG. 2 MICROSPECTROPHOTOMETER OPTICAL SYSTEM



MICROSPECTROPHOTOMETER ELECTRONIC BLOCK DIAGRAM

Fig

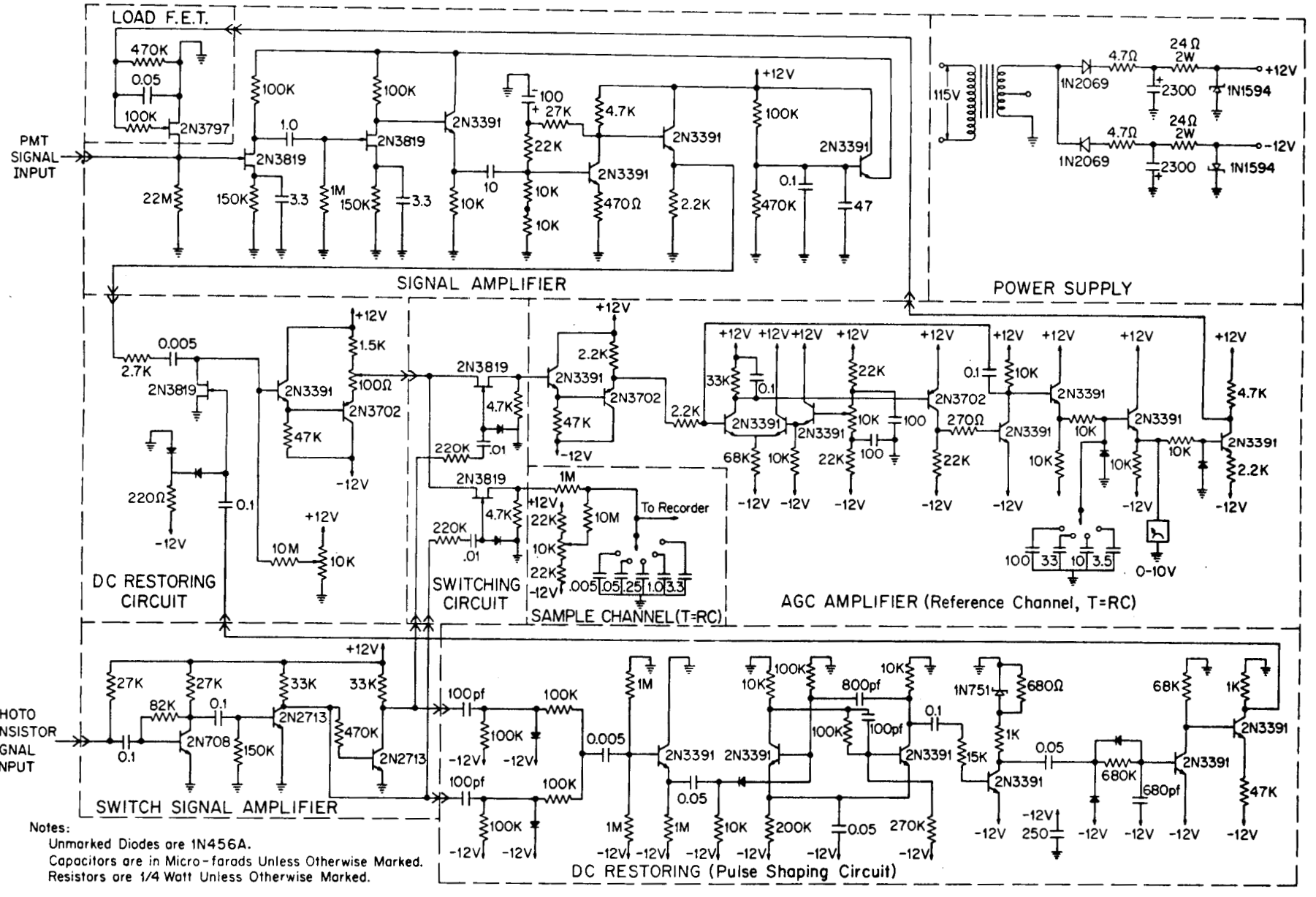
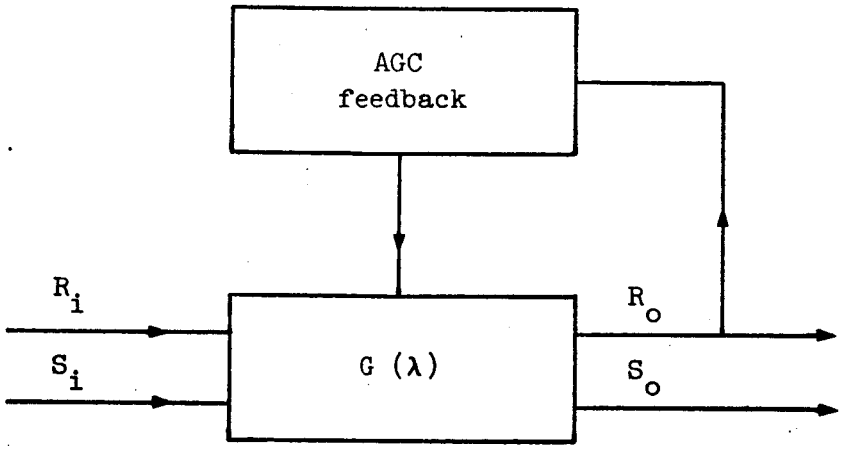

LOAD F.E.T.
PMT SIGNAL INPUT
SIGNAL AMPLIFIER
POWER SUPPLY
115V
1N2069
1N1594
+12V
-12V
DC RESTORING CIRCUIT
SWITCHING CIRCUIT
SAMPLE CHANNEL (T=RC)
To Recorder
AGC AMPLIFIER (Reference Channel, T=RC)
0-10V
PHOTO TRANSISTOR SIGNAL INPUT
SWITCH SIGNAL AMPLIFIER
DC RESTORING (Pulse Shaping Circuit)
Notes:
Unmarked Diodes are 1N456A.
Capacitors are in Micro-farads Unless Otherwise Marked.
Resistors are 1/4 Watt Unless Otherwise Marked.

Fig. 5



$$R_i \propto I(\lambda)\, A(\lambda)$$

$$S_i \propto I(\lambda)\, A(\lambda)\, S(\lambda)$$

$$\therefore R_o \propto I(\lambda)\, A(\lambda)\, G(\lambda)$$

If feedback is arranged to keep

R_o constant, then

$$G(\lambda) \propto \frac{I}{I(\lambda)\, A(\lambda)}$$

$$\therefore S_o \propto I(\lambda)\, A(\lambda)\, S(\lambda)\, G(\lambda) = S(\lambda)$$

Fig. 6

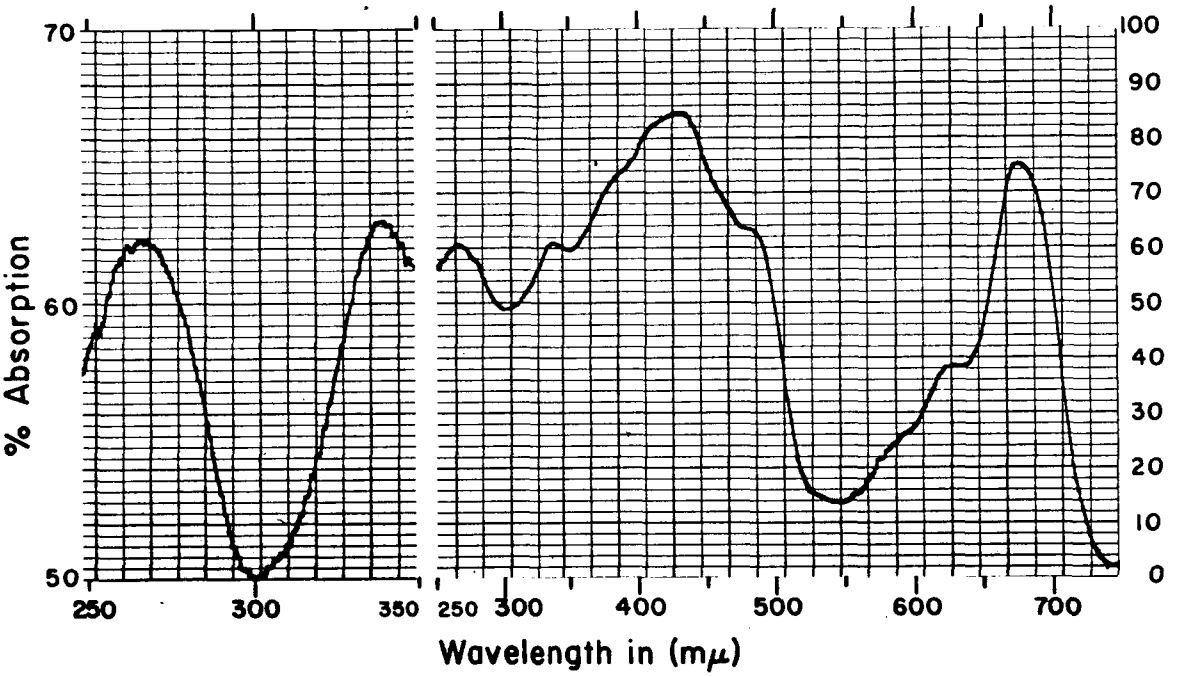

70
60
50
% Absorption
250
300
350
250
300
400
500
600
700
100
90
80
70
60
50
40
30
20
10
0
Wavelength in (mμ)

Fig. 7

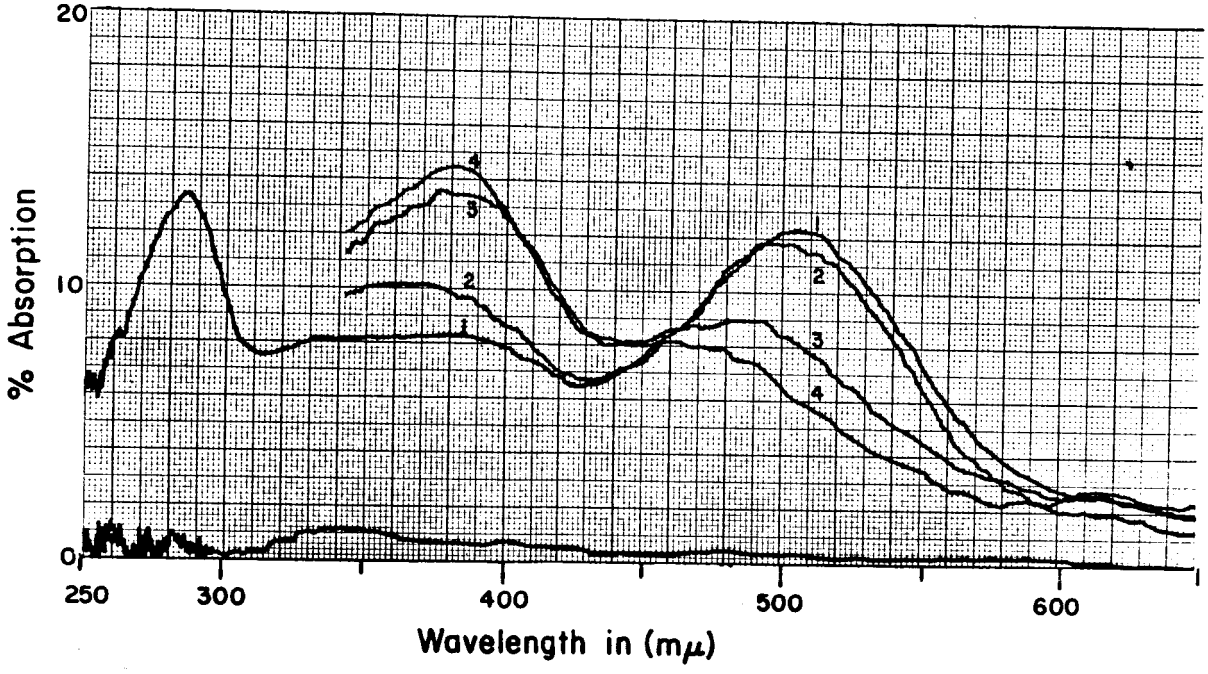

20
10
0
% Absorption
250
300
400
500
600
Wavelength in (mμ)
1
2
3
4
1
2
3
4

Fig. 8

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